Computational Studies of the Histamine H4 Receptor-Ligand Interactions

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Chapter 7

Summary, conclusions, and perspectives

1. Summary

The research described in this thesis makes use of current computational drug discovery approaches to better understand ligand-receptor interaction and develop novel compounds that target the histamine H_4 receptor (H_4R). This histamine receptor subtype was discovered in 2000,[1-7] and is reported to play an important role in the pathology of inflammatory-related diseases such as asthma, allergic rhinitis and pruritis.[8-11] Considering these promising applications, the H_4R has attracted academia and industry to join in the drug discovery quest to target this receptor.[12-14] The combined efforts result in a wealth of H_4R-related information, including the molecular biology of the receptor and the characteristics of the H_4R ligands. **Chapter 1** gives an overview of the known H_4R ligand scaffolds and their structure-activity relationships (SAR). This ligand-based information is integrated with three-dimensional protein models that are constructed using homology modeling and site-directed mutagenesis (SDM) studies. This results in a detailed structural and molecular understanding of H_4R-ligand interactions.

In **chapter 2** the scope of this thesis is defined. The studies intent to use modern computational medicinal chemistry approaches to analyze and integrate both ligand-based and protein-based information in order to unravel the molecular features that are important for H_4R-ligand interactions. The acquired understanding and generated molecular models can be used to find new H_4R ligands by virtual screening protocols and to guide H_4R hit optimization efforts.

In **chapter 3** a quantitative structure-activity relationship (QSAR) model of a series of quinozaline-sulfonamide-containing H_4R ligands is generated. A wide variety of molecular descriptors of these H_4R ligands are calculated and the properties that correlate best with the H_4R binding affinity are identified. The QSAR model has good predictive ability for the affinity of quinazolines with variations in the sulfonamide moiety. The studies indicate that the H_4R pocket that binds the sulfonamide substituents is rather small but tolerates a variety of substituents. It is noted that the model fails to predict the affinity of a compound that is structurally different from the ligands that are used in the training set. This finding indicates limitations of the QSAR model.

Chapter 4 presents the generation of QSAR models of clobenpropit analogs as dual active ligands for H_3R and H_4R. The clobenpropit analogs are useful tools to identify ligand descriptors that are important for H_4R and H_3R binding. The set of descriptors is different for the two histamine receptor subtypes, reflecting the subtle differences in the ligand binding of these homologous proteins. The QSAR models indicate that exploring energy-related descriptors can be useful to design selective ligands for the H_4R. The descriptor that is best able to distinguish H_3R and H_4R affinity is **E_stb,** the bond stretch-bend cross-term potential energy descriptor. This indicates that the ligand binding pocket of the H_4R is tighter than that of the H_3R. The set of selected descriptors of the QSAR models provide ligand-based information to construct the three-dimensional computational models of both H_3R-ligand interactions and H_4R-ligand interactions.

In **chapter 5**, the molecular determinants that drive H_3R over H_4R selectivity are identified by combining ligand-based QSAR models, protein-based H_4R modeling studies, and *in silico* guided site-directed mutagenesis experiments. This information is subsequently used to elucidate the binding modes of clobenpropit and its analogues in the H_4R binding pocket. In line with **chapters 3** and **4**, QSAR studies indicate that the descriptors that are related to ligand size and conformational energy are correlated with H_4R binding affinity. These 2D-QSAR models are followed by 3D-QSAR studies that identify the molecular interaction probes that determine H_3R over H_4R selectivity. Linking these probes to specific residues in H_4R receptor models has guided site-directed mutagenesis studies to elucidate H_4R-ligand binding modes. The studies indicate that clobenpropit can adopt two distinct binding modes in H_4R, while the addition of a cyclohexyl group to the clobenpropit isothiourea moiety allows the ligand to adopt only one specific binding orientation in the H_4R binding pocket. This three-dimensional protein-ligand interaction models are supported by ligand-based experimental data and give new insights into ligand recognition by H_4R. This experimentally driven modeling approach can also be used to elucidate the structural detail of other protein-ligand complexes.

The H_4R protein models that were developed and refined in the course of these PhD studies were also used in drug discovery efforts. The construction of the structure-based virtual screening (SBVS) protocols to identify new H_4R ligands is presented in **chapter 6**. The SBVSs are validated by retrospective analyses and subsequently employed to screen fragments. The results show that the SBVS protocols are comparable to 2D-LBVS in recognizing active H_4R fragments, but the structural diversity of the hits is higher

when using the SBVS protocols. In the prospective screening campaigns, the SBVS protocols are validated as a powerful tool for identifying new active H_4R fragments from commercially available screening collections.

2. Conclusions and perspectives

Although the histamine H_3R and the H_4R have very different (patho)physiological roles, the proteins are highly homologous in terms of amino acid sequence similarity in the putative ligand binding pocket in the transmembrane (TM) helical domain (~85% identity and ~56% identity)[9, 15, 16] and ligand binding profiles.[15, 17] The first compounds that were shown to have activity for H_4R were histamine derivaties (i.e., imidazole-containing H_3R reference compounds), including clobenpropit and its analogues that are presented in **chapter 4**. This dual activity reflects the similarity of the binding pockets and indicates that it is a challenge to design H_3R ligands with selectivity over the H_4R, and vice versa. Notably, there are subtle differences in the affinity at different subtype receptors that warrant further investigation. These subtle differences might enable the identification of molecular features of the histaminergic ligands that are important for distinguishing between H_3R- and H_4R-ligands interactions. The tools and techniques in computer-aided drug discovery (CADD) have made significantly progress, enabled by the advances in computing power that increases exponentially.[18, 19] These tools, e.g. QSAR, molecular dynamics, similarity searches, and molecular docking, are widely used and bring more rational design to medicinal chemistry programs.[18] The applicability of these techniques to investigate the molecular features of ligand-H_4R interaction and for H_4R drug discovery is therefore of considerable and timely interest.

Having reviewed the published H_4R compounds and highlighting the ligands with some H_4R selectivity over the H_3R, a pharmacophore model was proposed (Fig. **15** in **chapter 1**). This pharmacophore model comprises of 2 hydrogen bond donors, 1 aromatic heterocycle or small polar group, and 2 lipophilic or aromatic moieties. This model, however, is very similar to the one previously proposed for H_3R ligands.[17] The H_4R pharmacophore model summarizes the nine published H_4R scaffolds (i.e., histamine derivatives, guanidines, isothioureas, guanidine-isothioureas, dibenzodiazepines, indole-carboxamides, aminopyrimidines, quinoxalines and quinazolines) that were identified by 2010. The number of H_4R ligands is still growing. The number of publication that describes H_4R research is still increasing throughout the years (Fig. **1**). In 2010, one

novel ligand was identified, i.e. the benzofuro[3,2-d]pyrimidine-containing hit (**1**). This compound was identified by a ligand-based virtual screening (LBVS) campaign that employed JNJ7777120 (**1**) as the reference H_4R compound.[20] Not surprisingly, this novel compound fulfills the pharmacophore, as JNJ7777120 was a prominent structure in the construction of the model. Interestingly, the optimized H_4R ligand in this study has a considerable selectivity over other histamine receptors.[20] This success story provides strong indication of the applicability of the CADD techniques in the drug discovery targeting the H_4R.

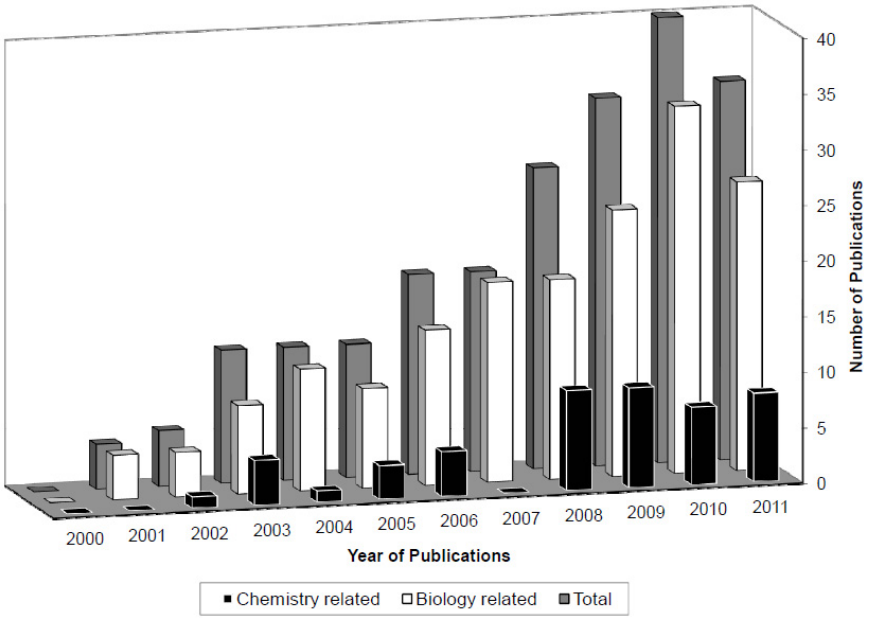

Fig. 1. Number of H_4R-related publications (status: December 4th, 2011).

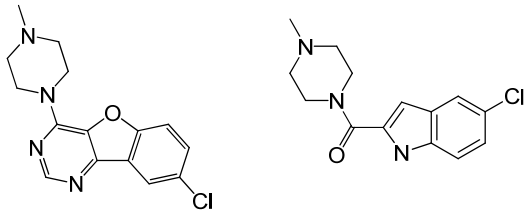

Fig. 2. Structures of the hit discovered in an LBVS campaign (**1**) and JNJ7777120 (**2**).[20, 21] JNJN7777120 was the reference in the LBVS campaign.[20]

This thesis describes the use of ligand-based data in combination with structure-based data. This enables linking the H_4R ligands features to the H_4R protein properties, thereby pinpointing H_4R-ligand interactions (Fig. **3**). The optimized homology models for H_4R enables the construction of SBVS protocols to identify novel H_4R ligands. These approaches can be updated and improved if new ligand binding data and selectivity information becomes available.

The main CADD approaches used in the research described in this thesis are QSAR, homology modeling, similarity searches and molecular docking. It is shown in this thesis that these approaches can help to guide medicinal chemistry and drug discovery efforts. In a rough outline, the approach we used starts with the available ligand screening data (described in subsection 2.1) to come to a better understanding of receptor binding, then uses the ligand-based models to come to improved and validated homology models for the receptor (subsection 2.2). Finally, the combined understanding is used in virtual screening studies to identify H_4R binding ligands (subsection 2.3)

2.1. Ligand-based quantitative structure-activity relationships (QSAR) studies on quinazoline sulfonamides and clobenpropit analogs

Ligand-based drug design can be complicated when having to use data resulting from different assays and different sources. For our H_4R studies, we were in the fortunate position to have available substantial data that was all generated in house. Therefore, the data was considered a good premise to perform QSAR studies in order to generate models that can give more insights into the properties that are important for receptor binding, and that can also be used as a tool to predict the H_4R affinity of a compound.

A QSAR model of quinazolines sulfonamides was successfully generated (**Chapter 3**). The model was validated with a test set and can be defined as a predictive model according to criteria introduced by Golbraikh and Tropsha.[22] However, the model fails to predict one particular compound in the test set. The compound has one atom, i.e. iodine, that none of the compound in the training set has. This illustrates a general concern in QSAR studies and indicates the limitations with regards to the domain of applicability[23] of this particular QSAR model. The model has 6 descriptors to explain 31 compounds in the training set. This ratio of 1:5 is common in a QSAR model, although it has been

described that this ratio can lead to over-fitting of the model.[24] This study also raises the difficulty of mechanistic interpretation of the model, as many of the descriptors that have been developed over the years do not describe "simple" physicochemical properties[23] As one of the aims of this research was to guide the medicinal chemistry efforts, the mechanistic interpretation of the models was considered an important achievable. Despite difficulties to interpret the model, the study led us to acknowledge the importance of diversity, steric and electronic properties. This indicates that the H_4R pocket has important steric constraints for the ligands to adapt to interact with the receptor.

Other useful ligand-based information was obtained from clobenpropit analogs. These compounds have dual activity as they interact with both H_3R and H_4R. Clobenpropit itself was reported as an inverse agonist at H_3R and as an agonist at H_4R.[25, 26] Studying these series of compounds allowed us to identify features that are important for affinity and also for selectivity. So far, we have not been successful in determining the features that are responsible for functional activity, clearly indicating that our understanding of the GPCR activation mechanism on a molecular level still needs to be improved. *In silico* predictions of ligands' functional activity are indeed still considered very difficult.[27-29] Employing the same QSAR modeling approaches as described for the quinozaline sulfonamides, cross-target QSAR models of clobenpropits were derived (**chapter 4**). The models have a good internal validation, but have not been externally validated due to the limited number of data points. The results clearly show that within this series, designing H_4R compounds with selectivity over H_3R is possible. In the mechanistic interpretation of the models, we found similar difficulties as presented in the previous paragraph since we used the same complex theoretical descriptors. Nevertheless, an energy-related descriptor was identified as the property that can induce selectivity for H_3R or H_4R. This descriptor indicates that the binding H_4R pocket is more space limited, even compared to the H_3R binding pocket. Encouragingly, a similar descriptor was found to be important to describe the H_4R affinity of the quinazoline sulfonamide ligands, as indicated in the previous paragraph and in **chapter 3**. However, as indicated earliler, generating QSAR models to predict functional activity of these compounds for either H_3R or H_4R still remains challenging. Notably, recently published prospective SBVS on inverse agonist bound H_1R crystal structure reported that some H_1R agonists were also discovered.[27] Moreover, the first reported non-imidazole neutral H_4R antagonist JNJ7777120,[21] which later has become a reference ligand to perform some VS studies[20] and to generate 3D models of H_4R-ligand interactions,[30-32] was reported that its

functional activity is in fact biased.[13, 33] It acts as a neutral antagonist in G-protein-dependent signaling,[21] but shows partial agonistic activity in β-arrestin-2 recruitment.[13, 33]

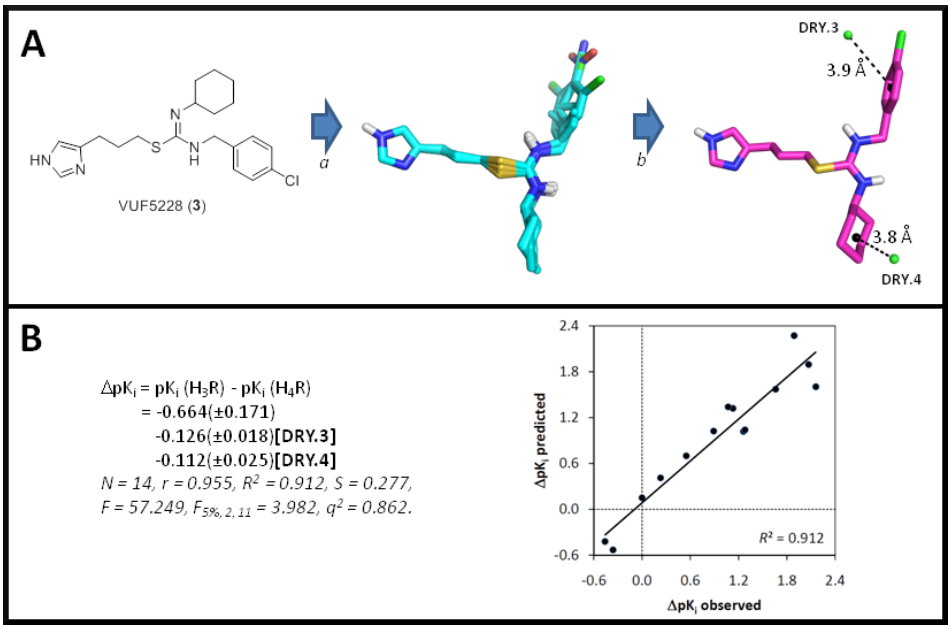

Fig. 3. Scheme of 3D-QSAR approaches identifies the H_4R-ligand interaction points. [a]3D structure generation of the reference compound (VUF5228 (**3**)) and alignment of compounds in the training set.[b]MIF-based 3D-QSAR results in the identification of the H_4R hydrophobic interaction points (**A**). The generated QSAR model and the graph between observed and calculated ΔpK_i (**B**).

To overcome the difficulties in the interpretation of the QSAR models, we incorporated 3D-QSAR approaches, i.e., by using molecular interaction fields (MIF)-based probes as the descriptors (**Chapter 5**). Next to revealing important properties we also wished to identify the binding points, to which these properties are related. This understanding allows the interpretation and fine-tuning of receptor homology models. To enable these studies, new clobenpropit analogs with two lipophilic moieties attached to the isothiourea group in the imidazole side-chain were designed and synthesized. The compound series were designed to fulfill the proposed H_4R pharmacophore model (Fig. **3** in **chapter 1**) while occupying most of the available space in the H_4R binding pocket. By doing this, the number of alternative binding modes for these ligands in the site is drastically reduced. Interestingly, these novel compounds have dual H_3R and H_4R activity but significant

selectivity can be obtained. In order to construct the models, the high affinity compound VUF5228 (**3**) (Figures **3**) was selected as the reference ligand that was used for aligning the other compounds in this study. Using the default setting of CORINA resulted in two different 3D configurations of VUF5228 (**3**). *Ab initio* calculation assisted the selection of the most favorable 3D configuration of VUF5228 (**3**). Probing the environment of the aligned ligands resulted in the identification of descriptors types that are in line with the results of the 2D-QSAR described in **chapters 3-5**. The descriptors in the 3D-QSAR model indicate a more pronounced role for hydrophobic properties of the ligands. Furthermore, the studies give a localization of the interaction site points of the H_4R (Fig. **3**). These interaction points can be linked to properties of specific amino acid residues of the H_4R protein ($N^{4.57}$, $E^{5.46}$, $T^{6.55}$ and $Q^{7.42}$)[30] and thereby support homology modeling, resulting in refined structural models.

2.2. Modeling the H_4R-ligand interactions

Besides characterizing the H_4R ligands, the ligand-based QSAR approaches have important consequences for homology modeling strategies. Detailed knowledge of the 3D structure of the H_4R can provide important insights into receptor-ligand interactions and can be used for the discovery of new ligands. Homology modeling needs a 3D structure of a homologous protein as a template. Ideally high resolution crystal structures are used as templates. Unfortunately, the H_4R is a G-protein coupled receptor (GPCR),[7] for which available crystal structures have been very limited.

At the start of this research in early 2008, the only template for homology modeling of the H_4R was bovine rhodopsin (bRho).[34, 35] In an exciting and very recent development, more crystal structures of human GPCRs have been solved, i.e. the beta adrenergic receptors type 2 (ADRB2), the A2A adenosine receptor (AA2AR), the dopamine D3 receptor (DRD3), the CXCR4 and the histamine H_1 receptor (H_1R).[36-41] The availability of these GPCR crystal structures, notably the inverse agonist and antagonist bound structures of ADRB2, provides opportunities for the construction of high resolution H_4R models, that can explain and predict H_4R-ligand binding.[31] The most recent one, the crystal structure of H_1R bound to an inverse agonist doxepin is very closely related to the H_4R (~73% similarity in the putative binding pocket; see Fig. **17** in **chapter 1**). It offers new opportunity to model the H_4R-ligand interactions more accurately.

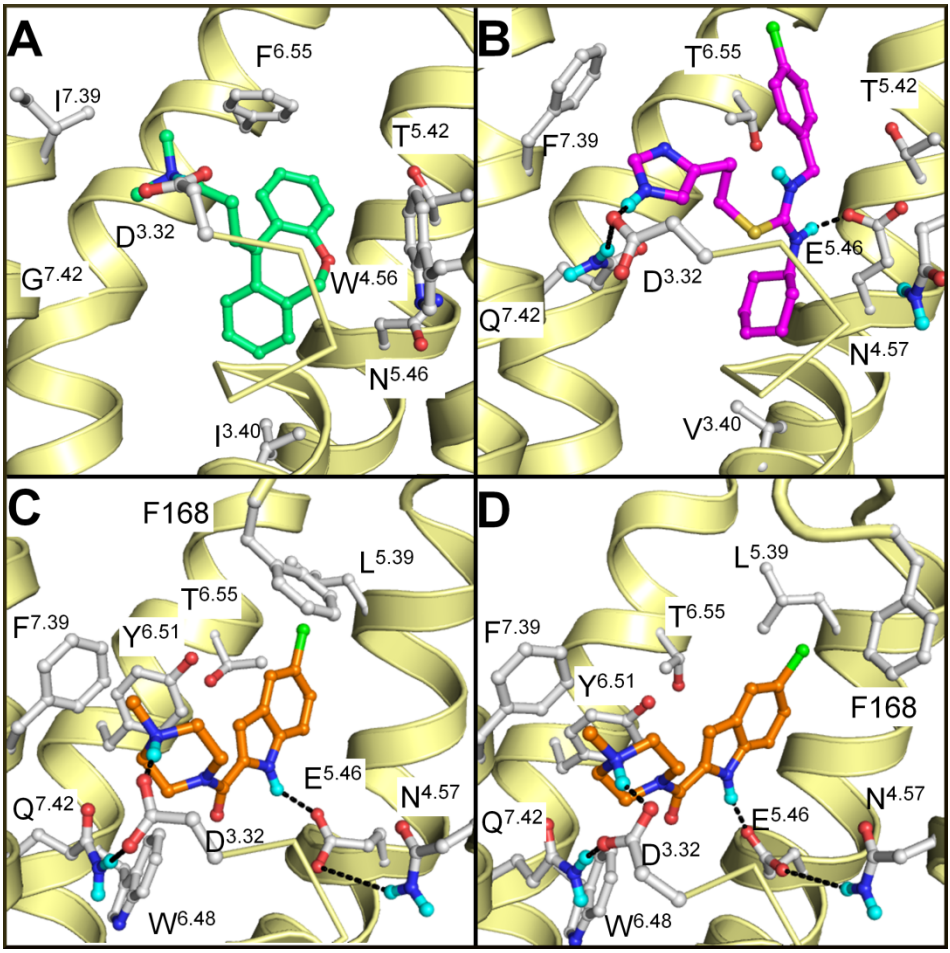

Fig. 4. Comparison of ADRB2-carazolol crystal structure based H_4R models (**B**, **C**) and a H_1R-doxepin (light green) crystal structure (**A**) based H_4R model (**D**). H_4R are constructed with JNJ7777120 (**2**, orange carbon atoms, **C** and **D**) and VUF5228 (**3**, magenta carbon atoms, **B**). The backbone of TM helices 4, 5, 6, and 7 are represented by yellow ribbons and part of TM3 is shown as ribbon (the top of the helix is not shown for clarity). In **C** and **D**, small part of ECL2 is shown to show the main differences. Important binding residues identified previously in **chapter 5** (**A**, **B**) and **6** (**C**, **D**) are depicted in grey.

Following the results of the 3D-QSAR studies, H_4R homology models were generated using the ADRB2 (pdb code: 2RH1) as the best template that was available at that time. Clobepropit (**4**) and VUF5228 (**3**) were docked in the putative binding pocket guided by previous H_4R modeling strategies and using data from site directed mutagenesis studies.[31, 34, 42] Thus, two H_4R H-bond acceptors ($D^{3.32}$ and $E^{5.46}$) were used as the main H_4R-ligand interaction points.[31, 34, 42] Since the ligands have two basic groups, and

therefore two H-bond donors, there are two corresponding binding poses of that both fulfill the key interaction requirements (Fig. **5** in **chapter 5**). Therefore we used the dual H_3R and H_4R activity features of the clobenpropit series to interrogate other important interaction points of the H_4R pocket.

By linking the emerged important MIF probes of the 3D-QSAR studies with the protein homology models we proposed site-directed mutagenesis studies to elucidate the binding mode of clobenpropit and its analogues in H_4R. The binding affinities of clobenpropit (**4**) and VUF5228 (**3**) at the $E^{5.46}Q$ mutant and the five H_4R mutants mimicking the H_3R binding pocket ($V^{3.40}A$, $N^{4.57}Y$, $T^{5.42}A$, $T^{6.55}M$, $Q^{7.42}L$) were examined. The previously reported H_4R $E^{5.46}Q$ mutant has interesting ligand dependent effects on binding affinity.[31, 34, 42] The H_4R $E^{5.46}Q$ mutant decreases the affinity of histamine, VUF8430, and clobenpropit but does not affect the affinity of clozapine , JNJN7777120, and VUF5338.[31, 34, 42] Furthermore, 3 out of 5 newly designed mutants have significant effects on the affinities of either clobenpropit (**4**) or VUF5228 (**3**). By examining the location of affecting mutations, the binding pose of clobenpropit (**4**) and VUF5228 (**3**) could be identified. Clobenpropit (**4**) can adopt two distinct binding modes in H_4R, while VUF5228 (**3**) adopts only one specific binding orientation in the H_4R binding pocket. This ligand-guided protein modeling that is supported by pharmacology and molecular biology experiments leads to an improved understanding of ligand-receptor interaction.

During preparation of this manuscript, the H_1R crystal structure (pdb code: 3RZE),[40] became available (Fig. **4A**). Binding site analysis of this new structure indeed identifies the H-bond between the co-crystallized ligand doxepin with $D^{3.32}$. Aligning the crystal structure with the ADRB2-based H_4R homology models generated in **chapter 5** shows that VUF5228 (**3**) (Fig. **4B**) occupies a similar hydrophobic binding pocket in the H_1R crystal structure. Both doxepin and VUF5228 show interaction to the hydrophobic residue in the position 3.40 ($I^{3.40}$ and $V^{3.40}$ for H_1R crystal structure and H_4R model, respectively) (Fig. **4A** and **B**). How a ligand occupies this pocket in H_4R plays an important role in the affinity of the ligand at H_4R and its selectivity over H_3R.[30, 32]

Homology modeling of the H_4R based on this new H_1R crystal structure represents an interesting possibility for future research. Fig. **4C** and **D** show ADRB2-carazolol crystal structure based and H_1R-doxepin crystal structure based H_4R models, respectively. The TM binding pocket is very similar in both H_4R models and the differences are found in the second extracellular loop (EL2), which slightly affect the orientation of ligands. In the

ADRB2-based the chlorine atom of JNJ7777120 (**2**) is located between EL2 (F168), TM5 ($L^{5.39}$), and TM6 ($T^{6.55}$), while in the H_1R-based model the chlorine atom of JNJ7777120 is accommodated between TM5 ($L^{5.39}$ and $T^{5.42}$) and EL2 (F168). While the TM fold of the ADRB2 and H_1R crystal structures are indeed very similar,[38, 43] the different EL2 loop conformations (in particular the orientation of F168) results in different H_4R models. These subtle differences in both binding pocket structure and reference ligand binding mode result in relatively small differences in retrospective VS accuracies.

2.3. The applicability of the H_4R homology models in virtual screening campaigns

Having developed H_4R homology models (**chapter 5**) enables the construction and use of SBVS protocols to discover new H_4R ligands. Most of the optimized H_4R ligands to date come from high-throughput screening (HTS) campaigns and classical medicinal chemistry programs.[9, 12, 14] Only few efforts to use structure-based virtual screening campaigns to discover new H_4R ligands have been reported in literature and these resulted in a limited number of hits with IC_{50} values in the micromolar range.[44]

We recently screened a fragment library against the H_4R. The resulting data, consisting of active and inactive H_4R fragment information was used to validate the applicability of the SBVS protocols to find fragments for the H_4R, retrospectively (**chapter 6**). These structure-based fragment screening campaigns are challenging for a number of reasons. Docking of the small fragments into a large binding pocket introduces complications with sampling (i.e., many different binding possibilities). Accurately ranking the different binding modes is difficult as scoring functions have not yet been optimized for the ranking/prioritization of fragment docking poses. With regards to hit identification it is noted that fragments often have low affinity making it more difficult to detect hits using the standard assays. One main advantage in the discovery of new fragments is the ability to escape from the so-called “privilege scaffold” for the H_4R ligands, as was shown by Smits et al.[14] The SBVS protocols that we established were retrospectively benchmarked with ligand-based virtual screening (LBVS) protocols, both two-dimensional (2D) and three-dimensional (3D). The SBVS protocols showed comparable ability with 2D-LBVS in recognizing active H_4R fragments with more capacity in the discovery of structurally diverse H_4R fragment hits. The SBVS protocols were

subsequently used to find new active H_4R fragments (Fig. **6**) in a chemical database of commercially-available compounds.

The described protocols only constrain H-bonds of ligands to $D^{3.32}$. The previous study in **chapter 5**, as well as other studies,[31, 34, 42] has identified other molecular determinants in the ligand binding to the H_4R. This information has not explicitly employed in the present SBVS protocols. In future studies, retrospective SBVS experiments can be performed that vary the identified interactions points as a molecular docking constraint during the virtual screening experiments. These studies can lead to the validation of important interactions and lead to further refinement of the SBVS protocols. Similar to QSAR studies that are empowered by statistics, these optimization efforts can be more efficient and powerful by the assistance of factorial design approach, a statistics method commonly used for optimization by systematically examining the importance of the hypothesized important factors and interactions between them.[45, 46] The results can help pin point the factors that play an important role and how importance they are in a mathematical model.[46] In our case of the SBVS optimization, the factors are the H_4R plausible binding pocket residues that the H_4R ligands are identified to interact with.

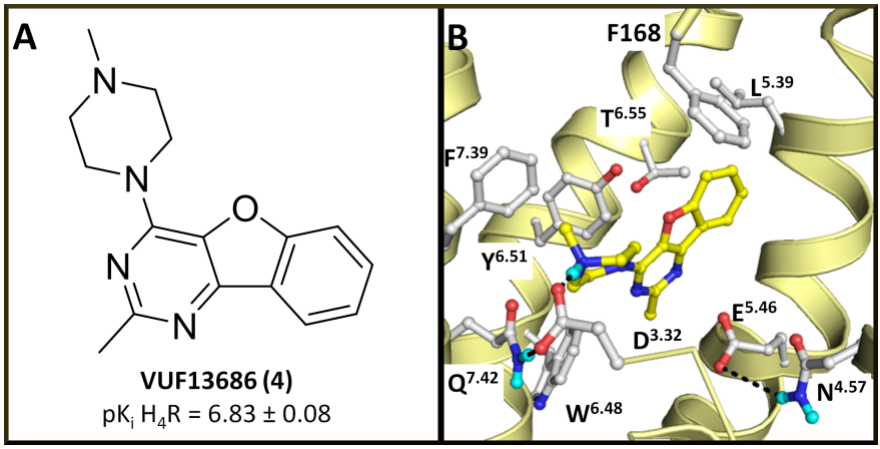

Fig. 5. One of the H_4R ligands in 2D (**A**) and in 3D (**B**, yellow carbon atoms; bound to ADRB2-carazolol crystal structure based H_4R model) which were discovered using SBVS protocols developed in **chapter 6**. Rendering of panel B is similar to Fig. **4**.

The increasing number of GPCR X-ray structures solved in the last few years[47-52] (and many more to be solved in the coming years)[53] will open up more opportunities to further push the limits of rational design and *in silico* discovery of small molecule ligands for GPCRs. Recent GPCR crystal structure-based virtual screening campaigns[47, 49, 50, 54, 55] indeed have yielded relatively high hit rates. More and detailed structural information will further increase the possibilities of GPCR structure-based identification and optimization of: i) receptor subtype selective ligands, ii) molecules with a specific functional effect; iii) ligands that target alternative (allosteric) binding sites, and iv) smaller molecules that are suitable starting points for fragment-based drug discovery (FBDD). The elucidation of the molecular determinants of receptor subtype selectivity can further be improved when crystal structures of closely related GPCRs become available,[28, 50, 56] while comparison of antagonist and agonist bound crystal structures[57] (will) give more insights into the detailed molecular mechanisms of GPCR activation and (selective) recognition of ligands with specific functional activity. The latest GPCR crystal structures furthermore for the first time provide ultimate proof that small molecule ligands can bind in different (allosteric) binding sites within the large GPCR binding pocket (i.e., in CXCR4)[41] and provide clues for additional allosteric sites close to the ligand bound orthosteric site (i.e., an anionic site in H_1R). **Chapter 6** demonstrates that structure-based virtual fragment screening against GPCR homology models (based on crystal structure templates) is already feasible, and a recent H_1R crystal structure-based virtual screening study suggests that higher resolution structures might even give higher hit rates of fragment-like ligands. FBDD approaches *combining* structure-based *in silico* ligand design with synthesis and molecular pharmacology will however be needed to target new (allosteric) GPCR binding sites (by, e.g., fragment growing, merging, or linking).[58, 59] The work presented in the current thesis shows how combined and experimentally guided *in silico* modeling approaches can be used for the elucidation of the molecular determinants of GPCR ligand binding. These integrative approaches can serve as a blueprint for the (fragment-based) design of receptor subtype selective GPCR ligands (that fit specific binding sites and/or have specific functional effects) in the future.

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